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# Agonist-stimulated high-affinity GTPase in *Dictyostelium* membranes

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GTP hydrolysis in *Dictyostelium discoideum* membranes is caused by a low ($K_m > 1$ mM) and a high affinity ($K_m$ 6.5 $\mu$M) GTPase. cAMP enhances GTP hydrolysis apparently by increasing the affinity of the high affinity GTPase (stimulated $K_m$ 4.5 $\mu$M); the low affinity GTPase was not affected by cAMP. Stimulation of GTP hydrolysis by cAMP was maximal at early time points and declined thereafter. A half-maximal stimulation of GTPase occurred at 3 $\mu$M cAMP and the specificity of cAMP derivatives for stimulation of GTPase activity showed a close correlation with the specificity for binding to the cell surface cAMP receptor. Treatment of *D. discoideum* cells with pertussis toxin decreased the cAMP-induced stimulation of GTPase from 42 ± 6% in control cells to 17 ± 9% in pertussis toxin-treated cells. These results suggest that the interaction of cAMP with its surface receptor leads to stimulation of high affinity GTPase in *D. discoideum* membranes. At least one of those enzymes may represent a guanine nucleotide-binding protein sensitive to pertussis toxin.

Enzyme activity; GTPase; GTP hydrolysis; cyclic AMP; Pertussis toxin; G-protein

## 1. INTRODUCTION

In the cellular slime mold *Dictyostelium discoideum* cAMP acts as a first and second messenger and is involved in chemotaxis [1], morphogenesis [2], and cell differentiation [3]. cAMP binds to highly specific surface receptors, which activate several enzymes, including adenylate cyclase and guanylate cyclase [4–6]. The produced cAMP is secreted [7] and relays the chemotactic signal to the more distal cells. The cGMP produced remains largely intracellular and is probably involved in the chemotactic reaction [8–10]. Prolonged stimulation of *D. discoideum* cells with constant cAMP concentrations induces desensitization of guanylate and adenylate cyclase within a few seconds and a few minutes, respectively [11–14].

In vertebrates the effector molecules are coupled to the surface receptors via signal transducing G-proteins [15–17]. These proteins not only bind guanine nucleotides but also hydrolyze GTP. When affected by hormone-activated receptors, GTP hydrolysis is increased, due to an increase in the turnover of these proteins from the inactive GDP-bound to the active GTP-bound states. The hormone-stimulated GTP hydrolysis in different systems is inhibited following ADP-ribosylation by cholera and/or pertussis toxin [15,18–21]. The existence of a G protein in *D. discoideum* membranes has been suggested previously by Leichtling et al. [22], who showed that a 42-kDa protein binds GTP and can be ADP-ribosylated by the cholera toxin.

Recent results [23,24] suggest the presence of fast and slowly dissociating forms of the cell surface cAMP receptor. cAMP induces the inter-

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*Abbreviations:* ATP$\gamma$S, adenosine 5′-*O*-(3-thiotriphosphate); AppNHp, adenylyl-imidodiphosphate; cAMP, adenosine 3′,5′-monophosphate; (Sp)-cAMPS, adenosine 3′,5′-monophosphorothioate, Sp-stereoisomer; 2′-dcAMP, 2′-deoxyadenosine 3′,5′-monophosphate; 8-Br-cAMP, 8-bromoadenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; DTT, dithiothreitol

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conversion of binding forms in vivo [25,26], which is promoted by guanine nucleotides in vitro [26–29]. This suggests the involvement of guanine nucleotide-regulatory proteins in chemosensory transduction. This view is further supported by the recent observation that guanosine triphosphates stimulate adenylate cyclase in vitro [30,31], and by the finding that treatment of cells with pertussis toxin affects activation of adenylate cyclase in vitro [31] and in vivo (Snaar-Jagalska, B.E., unpublished). Finally, cAMP increases the binding of [$^3$H]GTP to isolated membranes and at the same time accelerates the dissociation rate of bound [$^3$H]GTP [32].

An essential function of G-proteins is the agonist-stimulated hydrolysis of GTP. Therefore, we investigated whether the GTP hydrolysis is stimulated by cAMP in *D. discoideum*. The results show the presence of agonist stimulated high-affinity GTPase, which is partly sensitive to pertussis toxin.

## 2. EXPERIMENTAL

### 2.1. *Materials*

[$\gamma$-$^{32}$P]GTP (37.94 Ci/mmol) was purchased from New England Nuclear. cAMP, ATP, ATP$\gamma$S, AppNHp, (Sp)-cAMPS, GTP, creatine phosphate, creatine kinase and cAMP derivatives were obtained from Boehringer Mannheim. DTT was from Sigma. Pertussis toxin was purchased from List.

### 2.2. *Culture conditions and membrane isolation*

*D. discoideum* cells (strain NC-4) were grown as described in [13], harvested in 10 mM $KH_2PO_4/Na_2HPO_4$, pH 6.5, washed and starved in phosphate buffer by shaking at a density of $10^7$ cells/ml. After 5–6 h, cells were collected by centrifugation, washed twice with phosphate buffer, and the pellet was resuspended in 40 mM Hepes/NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7, to a density of $2 \times 10^8$ cells/ml. Homogenization was performed by pressing the cell suspension through a Nuclepore filter (pore size 3 $\mu$m) at 0°C. The lysate was centrifuged at $10000 \times g$ for 5 min, the pellet washed once with 10 mM triethanolamine-HCl, pH 7.4, containing 0.5 mM EDTA, and the final pellet resuspended in 10 mM triethanolamine-HCl, pH 7.4, to the equivalent of $1 \times 10^8$ cells/ml.

### 2.3. *GTPase assay*

GTPase activity of the *D. discoideum* membranes was determined with a reaction mixture containing [$\gamma$-$^{32}$P]GTP (0.1 $\mu$Ci/assay), 2 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM AppNHp, 0.1 mM ATP$\gamma$S, 10 mM DTT, 5 mM creatine phosphate (Tris salt), 0.4 mg/ml creatine kinase and 2 mg/ml bovine serum albumin (purified) in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 $\mu$l. After 5 min preincubation of the reaction mixture at 25°C, the reaction was initiated by the addition of 30 $\mu$l membranes (10–40 $\mu$g protein/tube) to 70 $\mu$l of a reaction mixture and conducted for 3 min, if not otherwise indicated. The reaction was terminated by the addition of 0.5 ml sodium phosphate buffer (50 mM), pH 2.0, containing 5% (w/v) activated charcoal. The reaction tubes were centrifuged at 4°C for 5 min at $10000 \times g$ and the radioactivity of 0.4 ml of the supernatant was determined using Cerenkov radiation.

Release of $^{32}P_i$ from [$\gamma$-$^{32}$P]GTP in the absence of membranes was 0.5–2.5% of added [$\gamma$-$^{32}$P]GTP. High-affinity GTPase was defined as the difference between total GTPase and low-affinity GTPase activity. Low-affinity GTPase activity was determined in the presence of 50 $\mu$M GTP [13,33], and was about 40–50% of total GTPase. Maximal hydrolysis of GTP did not exceed 10–15% of added GTP. GTPase assays were performed in triplicates, with intra-assay variation of less than 3% of the means. Experiments were repeated at least twice, with results comparable with those shown.

## 3. RESULTS

The aim of the present study is to investigate GTPase activity in *D. discoideum* membranes as a function of the interaction between cell-surface cAMP receptors and putative G-protein(s). To determine GTP-specific nucleoside triphosphatase a low concentration of GTP (0.25 $\mu$M) and maximal suppression of the non-specific nucleoside triphosphatases activity was used [33]. Addition of AppNHp, an inhibitor of a number of ATPases [34] decreased the rate of GTP hydrolysis from $101.0 \pm 4.2$ to $51.4 \pm 1.6$ pmol $P_i \cdot min^{-1} \cdot mg$ $protein^{-1}$ (table 1). Redistribution of radioactivity

Table 1

Hydrolysis of GTP in *D. discoideum* membranes by high-affinity GTPase

| Addition | [$\gamma$-$^{32}$P]GTP hydrolysis (pmol $P_i \cdot min^{-1} \cdot mg$ $protein^{-1}$) | |
|---|---|---|
| | Control | + cAMP |
| None | $101.0 \pm 4.2$ | $104.2 \pm 7.6$ |
| 0.2 mM AppNHp | $51.4 \pm 1.6$ | $55.1 \pm 2.2$ |
| 0.2 mM AppNHp + RS | $28.2 \pm 3.1$ | $32.5 \pm 1.0$ |
| 0.2 mM AppNHp + RS + 0.1 mM ATP | $18.2 \pm 0.7$ | $21.8 \pm 1.2$ |
| 0.2 mM AppNHp + RS + 0.1 mM ATP$\gamma$S | $11.8 \pm 1.2$ | $16.1 \pm 1.1$ |

Membranes were incubated with 0.25 $\mu$M GTP in the presence or absence of 3 $\mu$M cAMP for 10 min. The ATP regeneration system (RS) was 5 mM creatine phosphate and 0.4 mg/ml creatine kinase. The data are means of three experiments among guanine and adenine dinucleotides was prevented by a nucleoside triphosphate regeneration system and by ATP$\gamma$S. Under this condition the liberation of $^{32}P_i$ was suppressed to 6–8% and effectively stimulated by cAMP. The not easily hydrolysed derivatives of ATP, ATP$\gamma$S and AppNHp, were used to suppress production of cAMP by adenylate cyclase.

### 3.1. *Time course and kinetics of GTP hydrolysis*

The hydrolysis of GTP in *D. discoideum* membranes was multiphasic, and cAMP stimulated the initial hydrolysis of GTP (fig.1). Stimulation of $P_i$ release by 3 $\mu$M cAMP was routinely measured at 3 min of incubation because at this time point $P_i$ production and stimulation by cAMP were sufficiently large for accurate determination (inset of fig.1). The relationship between membrane protein and GTP hydrolysis was linear in the range of 10–40 $\mu$g membrane protein per assay for the incubation at 25°C for 3 min (not shown).

The hydrolysis of different concentrations of [$\gamma$-$^{32}$P]GTP in the absence and presence of 3 $\mu$M cAMP is shown in fig.2. Hydrolysis of [$\gamma$-$^{32}$P]GTP was potently reduced by increasing concentrations of unlabeled GTP (fig.2A). At all GTP concentrations below 50 $\mu$M, cAMP increased [$\gamma$-$^{32}$P]GTP hydrolysis (fig.2A). Both curves reached a plateau at about 50 $\mu$M GTP. These observations indicate that *D. discoideum* membranes contain a high-affinity, cAMP-sensitive GTPase and a low-affinity cAMP-insensitive GTPase ($K_m$ > 1 mM). The high-affinity GTPase exhibited an apparent $K_m$ value of about 6.5 $\mu$M (fig.2B). The stimula-

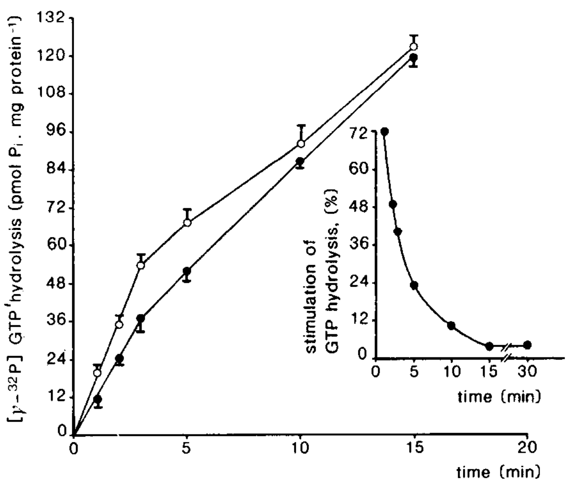


Fig.1. Time course of GTP hydrolysis in the absence and presence of cAMP. Membranes were incubated as described in section 2, but in a final volume of 1 ml. At the indicated times, aliquots of 100 $\mu$l were added to 500 $\mu$l of charcoal suspension. GTP hydrolysis was determined with 0.1 $\mu$M GTP in the absence (●) and presence (○) of 3 $\mu$M cAMP. Inset represents % stimulation of GTP hydrolysis by 3 $\mu$M cAMP.

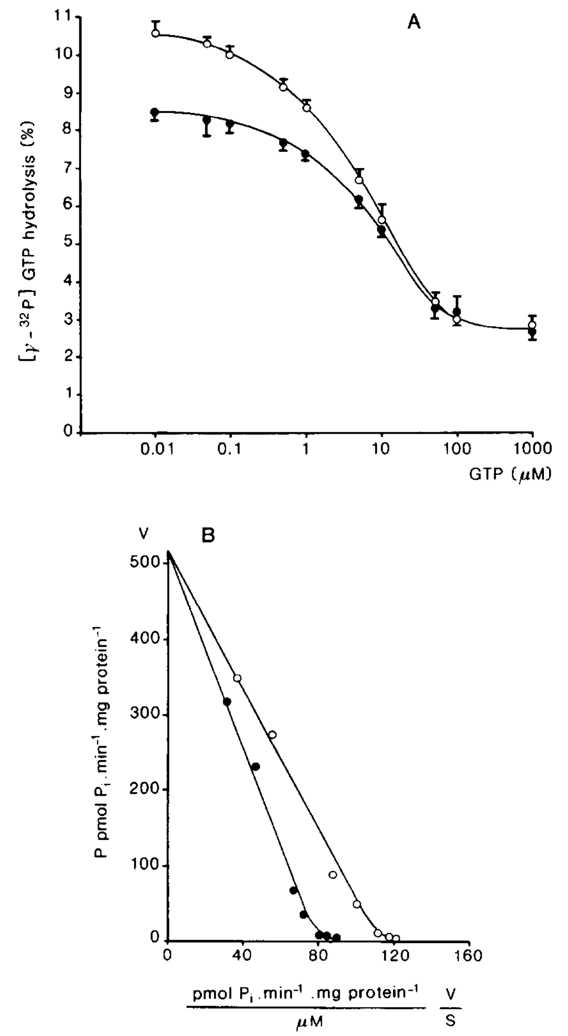


Fig.2. Kinetics of GTP hydrolysis in *D. discoideum* membranes. (A) Hydrolysis of [$\gamma$-$^{32}$P]GTP was determined at various concentrations of unlabeled GTP in the absence (●) and presence of 3 $\mu$M cAMP (○); (B) Eadie-Hofstee plot of a high-affinity GTPase. Low-$K_m$ GTPase was subtracted from the total GTPase activity and the apparent $K_m$ values (4.5 $\mu$M and 6.5 $\mu$M) of a high-affinity GTPase were extrapolated from the linear part of the curves.

tory effect of cAMP on GTP hydrolysis by the high-affinity GTPase occurred without a change in the $V_{max}$ value and was apparently caused by an increase of enzyme affinity for GTP from 6.5 $\mu$M to 4.5 $\mu$M (fig.2B).

### 3.2. *Agonist stimulation of GTPase activity*

The stimulatory effect of cAMP on GTP hydrolysis by the high-affinity GTPase in *D. discoideum* membranes was half-maximal at 3 $\mu$M cAMP and reached a maximum of 65% stimulation (fig.3). Specificity of cAMP derivatives for GTPase stimulation is shown in table 2. The order of GTPase stimulation was as follows: cAMP > 2′-dcAMP > (Sp)-cAMPS > 8-Br-cAMP, and cGMP and 5′-AMP were inactive. These derivatives bind to the chemotactic cAMP receptor with the same relative potencies [35], which is quite different from the binding specificity of cAMP-dependent protein kinase [36], indicating a functional coupling between the cell-surface cAMP receptor and a high-affinity GTPase.

### 3.3. *Influence of pertussis toxin on cAMP-stimulated GTP hydrolysis*

The effects of pertussis toxin treatment in vivo on the GTPase(s) activity in the membranes are shown in table 3. GTPase activity was measured at 0.01, 0.1 and 1 $\mu$M. After pertussis toxin treatment the basal GTPase activity was as in control membranes at all GTP concentrations (NS, $p > 0.5$). cAMP stimulated GTPase activity at 0.01 $\mu$M GTP by 42 ± 6%. This stimulation was significantly (*, $p < 0.01$) reduced by pertussis toxin treatment to 17 ± 9%. Similar results were obtained at higher GTP concentrations: no reduction of basal GTPase activity by pertussis toxin and a significant reduction of the cAMP-mediated stimulation of GTPase. Enhancement of GTPase by cAMP in membranes derived from pertussis toxin-treated cells was not completely lost ($p < 0.01$), suggesting that pertussis toxin inhibited at least one component of the high-affinity GTPase activity that is

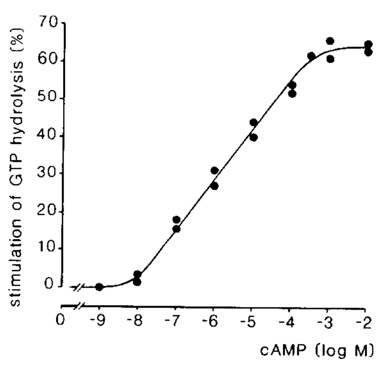


Fig.3. Concentration-response curve for cAMP stimulation of GTP hydrolysis. GTP hydrolysis by high-affinity GTPase was determined in *D. discoideum* membranes at the indicated concentration of cAMP with 0.1 $\mu$M GTP.

Table 2

Specificity of cAMP derivatives for stimulation of high-affinity GTPase in *D. discoideum* membranes

| Derivative | Stimulation of $P_i$ release (%) |
|---|---|
| No addition | 0 |
| cAMP | 53.2 ± 0.9 |
| 2′-dcAMP | 37.4 ± 2.4 |
| (Sp)-cAMPS | 29.9 ± 1.2 |
| 8-Br-cAMP | 12.2 ± 1.8 |
| cGMP | − 1.6 ± 2.1 |
| 5′-AMP | 1.4 ± 0.9 |

Membranes were incubated as described in section 2 with 0.1 $\mu$M GTP for 3 min in the presence or absence of 10 $\mu$M cAMP derivatives

Table 3

Effect of pertussis toxin on GTPase activity in *D. discoideum* membranes

| GTP ($\mu$M) | cAMP (3 $\mu$M) | GTPase activity (%) Control | | PT | |
|---|---|---|---|---|---|
| 0.01 | − | [a] 100 | NS | 101 ± 5 | [a] |
| 0.01 | + | 142 ± 6 | [a] | 117 ± 9 | |
| 0.1 | − | [a] 100 | NS | 101 ± 3 | [a] |
| 0.1 | + | 129 ± 4 | [a] | 108 ± 3 | |
| 1.0 | − | [a] 100 | NS | 100 ± 5 | [a] |
| 1.0 | + | 117 ± 4 | [a] | 107 ± 1 | |

*D. discoideum* cells were starved for 5 h in the absence or presence of 0.1 $\mu$g/ml pertussis toxin, washed and used for membrane preparation. GTP hydrolysis by high-affinity GTPase was determined in membranes of control and pertussis toxin-treated cells in the absence (−) and presence of 3 $\mu$M cAMP (+). Three concentrations of GTP, 0.01, 0.1 and 1.0 $\mu$M, were used. The results are means of three independent experiments normalized in each experiment to basal GTPase values (100%) in the control membranes. NS, the difference is not significant at $p > 0.5$; [a], the differences are significant at $p < 0.01$

coupled to the cAMP receptor. Alternatively, pertussis toxin was not used at a saturated concentration (see section 4).

## 4. DISCUSSION

In vertebrates GTPase activity associated with guanine nucleotide-binding protein leads to inactivation of the hormone-stimulated effector enzyme by hydrolysis of G-protein-bound GTP to GDP and $P_i$ [15,18–21].

In *D. discoideum* the existence of G-protein(s) has been suggested [23–31] but the stimulation of GTPase activity by receptor agonist was not observed. In this report we show the presence of high-affinity and low-affinity GTPase in *D. discoideum* membranes. Significant cAMP stimulation of a high-affinity GTPase could be detected only at low concentrations of GTP and by maximal suppression of the non-specific nucleoside triphosphatase activity. The specificity of GTPase stimulation by cAMP derivatives strongly supports the conclusion that cAMP interaction with a specific surface receptor leads to stimulation of a GTPase enzyme.

The present results, which characterise GTPase in *D. discoideum* membranes, are at least in part different from what has been observed in vertebrate cell membranes. The $K_m$ of the high-affinity GTPase in other cell membranes is in the range 0.2–0.6 $\mu$M GTP [18,19], while in *D. discoideum* it is 10–20-fold higher (6.5 $\mu$M). GTP inhibits cAMP binding to *D. discoideum* membranes also at 2 $\mu$M, which is also 10-fold higher than in vertebrates [27,37–39]. In vertebrate membranes hormonal agents increase the $V_{max}$ value of the enzyme without a major change in its substrate affinity [18,19], while in *D. discoideum* cAMP apparently stimulates the enzyme by increasing the affinity of the GTPase for its substrate GTP. This difference could be related to the long evolutionary distance between *D. discoideum* and vertebrate cells, and could be useful to elucidate the model of action of G-proteins.

In vertebrates pertussis toxin catalyses the ADP-ribosylation of a specific $G_i$ and blocks the inhibition of adenylate cyclase by GTP [15] and stimulation of GTPase by agonist [15,19]. In *D. discoideum* we have previously observed that inhibition of adenylate cyclase by GTP$\gamma$S was absent in membranes derived from pertussis toxin-treated cells [31]. The present observation that pertussis toxin treatment in vivo reduced stimulation of a high-affinity GTPase by cAMP supports our hypothesis that *D. discoideum* membranes contain $G_i$-like activity. The cAMP-stimulated effect was not completely lost after pertussis toxin treatment, suggesting that other G-proteins could be involved or that pertussis toxin was not used at a saturated concentration. The latter possibility seems unlikely since we have shown that treatment of cells with 100 ng/ml pertussis toxin completely abolished GTP inhibition of adenylate cyclase [31]. This raises the question about the nature and function of the other GTP-hydrolyzing protein which is affected by the cAMP receptor but not by pertussis toxin. It is possible that this GTP-hydrolyzing protein is involved in cAMP stimulation of adenylate cyclase [31] or phosphoinositide metabolism [40].

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